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We have investigated the interaction between Tiam1 and CD44 [the hyaluronic acid (HA) binding receptor] in breast tumor cells (SP1 cell line). Our data indicate that both the Tiam1 protein and the CD44v3 isoform are expressed in SP1 cells, and that these two proteins are physically associated as a complex in vivo. Using calmodulin-binding peptide (CBP)-tagged Tiam1 fragment [i.e. the NH2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain (designated as PHn-CC-Ex, aa393-aa738 of Tiam1] and an in vitro binding assay, we have detected a specific binding interaction between the Tiam1's PHn-CC-Ex domain and CD44. The binding of HA to CD44v3 of SP1 cells stimulates Tiam1-catalyzed Rac1 signaling and cytoskeleton-mediated tumor cell migration. Transfection of SP1 cells with Tiam1cDNA promotes Tiam1 association with CD44v3 and upregulates Rac1 signaling as well as HA/CD44v3-mediated breast tumor cell migration. Co-transfection of SP1 cells with PHn-CC-ExcDNA and Taim1cDNA effectively inhibits Tiam1 association with CD44 and efficiently blocks tumor behaviors. Taken together, we believe that the linkage between CD44v3 isoform and the PHn-CC-EX domain of Tiam1 is required for HA stimulated Rac1 signaling and cytoskeleton-mediated tumor cell migration during breast cancer progression.

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(5) INTRODUCTION

The ability of breast cancer cells to metastasize is known to correlate with changes in cytoskeleton-mediated tumor cell invasion and metastasis. A new invasion and metastasis-specific protein, Tiam1 (T lymphoma invasion and metastasis) has been shown to be closely associated with the cytoskeleton in breast cancer cells displaying both invasive and metastasis capability. Tiam1 was first identified as an oncogene due to its ability to activate Rho-like GTPases during malignant transformation. In this study we have investigated the interaction between Tiam1 and the metastasis-specific surface molecule, CD44 [the hyaluronic acid (HA) binding receptor] in breast tumor cells (SP1 cell line). A special emphasis has been placed on analyzing both structural and functional relationships between Tiam1 and CD44 isoforms such as CD44v3 in metastatic breast tumor cells. We believe that this new information concerning Tiam1-CD44v3 interaction has provided us a better understanding of Tiam1-mediated oncogenic signaling (e.g. RhoGTPase activation) and cytoskeleton function during extracellular matrix component (e.g. HA)-mediated metastatic breast tumor cell progression.

(6) BODY

SPECIFIC BACKGROUND

The transmembrane glycoprotein CD44 isoforms are all major hyaluronic acid (HA) cell surface receptors that exist on many cell types, including macrophages, lymphocytes, fibroblasts and epithelial cells (1-6). Due to its widespread occurrence and its role in signal transduction, CD44 isoforms have been implicated in the regulation of cell growth and activation as well as cell-cell and cell-extracellular matrix interactions (1-7). One of the distinct features of CD44 isoforms is the enormous heterogeneity in the molecular masses of these proteins. It is now known that all CD44 isoforms are encoded by a single gene which contains 19 exons (8). Out of the 19 exons, 12 exons can be alternatively spliced (8). Most often, the alternative splicing occurs between exons 5 and 15 leading to an insertion in tandem of one or more variant exons [v1-v10 (exon 6-exon 14) in human cells] within the membrane proximal region of the extracellular domain (8). The variable primary amino acid sequence of different CD44 isoforms is further modified by extensive N-and O-glycosylations and glycosaminoglycan (GAG) additions (9-12). In particular, CD44v3-containing isoforms have a heparin sulfate addition at the membrane-proximal extracellular domain of the molecule that confers the ability to bind heparin sulfate-binding growth factors (9,10). Cell surface expression of CD44v isoforms changes profoundly during tumor metastasis, particularly during the progression of various carcinomas including breast carcinomas (13-17). In fact, CD44v isoform expression has been used as an indicator of metastasis.

It has been shown that interaction between the cytoskeletal protein, ankyrin and the cytoplasmic domain of CD44 isoforms plays an important role in CD44 isoform-mediated oncogenic signaling (6,18,19). Specifically, the ankyrin-binding domain [e.g. "NGGNGTVEDRKPSEL" between aa 306 and aa320 in the mouse CD44 (20) and "NSGNGAVEDRKPSGL " aa304 and aa318 in human CD44 (21)] is required for the recruitment of Src kinase and the onset of tumor cell transformation (21). Furthermore, HA binding to CD44 stimulates a concomitant activation of $p185^{HER2}$-linked tyrosine kinase (linked to CD44s via a disulfide linkage) and results in a direct "cross-talk" between two different signaling pathways (e.g. proliferation vs motility/invasion) (22). In tumor cells, the transmembrane linkage between CD44 isoform and the cytoskeleton promotes invasive and metastatic-specific tumor phenotypes [e.g. matrix degradation (MMPs) activities, "invadopodia" formation (membrane projections), tumor cell invasion and migration] (23, 24). These findings strongly suggest that the interaction between CD44 isoform and the cytoskeleton plays a pivotal role in the onset of oncogenesis and tumor progression.

The Rho family (e.g. Rho, Rac and Cdc42) proteins are members of the Ras superfamily of GTP-binding proteins, structurally related to, but functionally distinct from Ras itself (25,26). They are associated with changes in the membrane-linked cytoskeleton (26). For example, activation of RhoA, Rac1 and Cdc42 have been shown to produce specific structural changes in the plasma membrane-cytoskeleton associated with membrane ruffling, lamellipodia, filopodia, and stress fiber formation (26). The coordinated activation of these GTPases is considered to be a possible mechanism underlying cell motility, an obvious prerequisite for metastasis (27-29). In particular, Rac1 activation is known to initiate oncogenic signaling pathways that promote cell shape changes (33,34), influence actin cytoskeleton organization (33,34) and stimulate gene expression (35-37). The question of whether Rac1 activation is also involved in CD44v3-related cytoskeleton function that results in the metastatic phenotypes (e.g. tumor cell migration) of breast tumor cells remain to be answered.

Tiam1 (T lymphoma invasion and metastasis) has been identified as an oncogene due to its ability to activate Rho-like GTPases during malignant transformation (38,39). Specifically, Tiam1 is capable of activating Rac1 *in vitro* as a guanine nucleotide exchange factor (GEF) and induce membrane cytoskeleton-mediated cell shape changes, cell adhesion and cell motility (34, 40-42). It also acts as a Rac-specific GEF *in vivo* and induces an invasive phenotypes in lymphoma cells (40). These findings have prompted several research groups to investigate the mechanisms involved in the regulation of Tiam1. For example, addition of certain serum-derived lipids [e.g. sphingosine-1 -phosphate (S1P) and LPA] to T-lymphoma cells promotes Tiam1-mediated Rac1 and Cdc42 signaling and T-lymphoma cell invasion (43). Tiam1 has also been found to be phosphorylated by protein kinase C (PKC) in Swiss 3T3 fibroblasts stimulated by lysophosphatidic acid (LPA) (44) and platelet-derived growth factor (PDGF) (45). Most recently, Exton and his co-workers demonstrate that phosphorylation of Tiam1 by Ca^{2+}/calmodulin-dependent protein kinase II (but not protein kinase C) regulates Tiam1-catalyzed GDP/GTP exchange activity *in vitro* (46). These findings support the notion that posttranslational modifications of Tiam1 by certain serine/threonine kinase(s) during surface receptor-mediated activation may play an important role in Tiam1-Rac1 signaling. Tiam1 transcript has been detected in breast cancer cells (39). However, it is not known whether there is any structural and functional relationship(s) between Tiam1-Rac1 signaling and CD44v3-mediated invasive and metastatic processes of breast cancer cells at the present time.

In this work, using a variety of biochemical, molecular biological and immunocytochemical techniques, we have found that the cell adhesion molecule, $CD44v_3$ isoform, which binds directly to hyaluronic acid (HA), is closely associated with Tiam1 [in particular, the NH2-terminal pleckstrin homology (PHn), a putative coiled coil region (CC) and an additional adjacent region (Ex)- designated as PHn-CC-Ex domain of Tiam1] in SP1 breast tumor cells. Most importantly, HA binding to $CD44v_3$ isoform stimulates Tiam1-specific GDP/GTP exchange for Rho-like GTPases such as Rac1 and promotes cytoskeleton-mediated tumor cell migration. These findings suggest that a transmembrane interaction between CD44v3 and Tiam1 plays an important role in promoting oncogenic signaling and tumor cell-specific phenotypes required for HA-mediated breast tumor cell migration.

MATERIALS AMD METHODS

Cell Culture: Mouse breast tumor cells (e.g. SP1 cell line) (provided by Dr.Bruce Elliott, Department of Pathology, and Biochemistry, Queen's University, Kingston, Ontario, Canada) were used in this study. Specifically, SP1 cell line was derived from a spontaneous intraductal mammary adenocarcinoma that arose in a retired female CBA/J breeder in the Queen's University animal colony. These cells were capable of inducing lung metastases by sequential passage of SP1 cells into mammary gland (47). These cells were cultured in RPMI1640 medium

supplemented with 5-7% fetal calf serum, folic acid (290mg/L), and sodium pyruvate (100mg/L).

COS-7 cells were obtained from American Type Culture Collection and grown routinely in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 1% glutamine, 1% penicillin and 1% streptomycin.

Antibodies and Reagents: . For the preparation of polyclonal rabbit anti-Tiam1 antibody or rabbit anti-CD44v3 antibody, specific synthetic peptides [≈15-17 amino acids unique for the C-terminal sequence of Tiam1 or the CD44v3 sequence] were prepared by the Peptide Laboratories of Department of Biochemistry and Molecular Biology using an Advanced Chemtech automatic synthesizer (model ACT350). These Tiam1-related or CD44v3-related polypeptides were conjugated to polylysine and subsequently injected into rabbits to raise the antibodies, respectively. The anti-Tiam1-specific or anti-CD44v3-specific antibody was collected from each bleed and stored at 4°C containing 0.1% azide. The anti-Tiam1 IgG or anti-CD44v3 IgG fraction was prepared by conventional DEAE-cellulose chromatography, respectively. Mouse monoclonal anti-HA (hemagglutinin epitope) antibody (clone 12 CA5) was purchased from Boehringer Mannheim. Mouse monoclonal anti-green fluorescent protein (GFP) was purchased from PharMingen. Escherichia coli (E. coli)-derived GST-tagged Rac1 was kindly provided by Dr. Richard A. Cerione (Cornell University, Itheca, NY).

Cell Surface Labeling Procedures: SP1 cells suspended in PBS were surface labeled using the following biotinylation procedure. Briefly, cells (10^7 cells/ml) were incubated with sulfosuccinomidobiotin (Pierce Co., Rockford, IL) (0.1mg/ml) in labeling buffer (150μM NaCl, 0.1M HEPES, pH 8.0) for 30 min at room temperature. Cells were then washed with PBS to remove free biotin. Subsequently, the biotinylated cells were used for anti-CD44v3-mediated immunoprecipitation as described previously (23). These biotinylated materials precipitated by anti-CD44v3 antibody were analyzed by SDS-PAGE, transferred to the nitrocellulose filters and incubated with ExtrAvidin-peroxidase (Sigma Co.). After an addition of peroxidase substrate (Pierce Co.), the blots were developed using Renaissance chemiluminescence reagent (Amersham Life Science, England) according to the manufacturers instructions.

Immunoprecipitation and Immunobloting Techniques: SP1 cells were solubilized in 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 buffer and immunoprecipitated using rabbit anti-CD44v3 antibody or rabbit anti-Tiam1 antibody followed by goat anti-rabbit IgG, respectively. The immunoprecipitated material was solubilized in SDS, electrophoresed and blotted onto the nitrocellulose. After blocking non-specific sites with 3% bovine serum albumin, the nitrocellulose filter was incubated with rabbit anti-Tiam1 antibody (5μg/ml) or rabbit anti-CD44v3 antibody (5μg/ml), respectively for 1h at room temperature followed by incubation with horse radish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution) at room temperature for 1 h. The blots were developed using Renaissance chemiluminescence reagent (NEN, DuPont, Boston, MA) according to the manufacturers instructions.

In some experiments, SP1 cells or COS cells [e.g. untransfected or transfected by various Tiam1 cDNAs including the full-length mouse Tiam1cDNA (FL1591) or HA-tagged NH_2-terminally truncated C1199 Tiam1cDNA or GFP-tagged PHn-CC-ExcDNA or C1199Taim1cDNA plus GFP-tagged PHn-CC-ExcDNA (as co-transfection) or vector only] were immunoblotted with mouse anti-HA antibody (5μg/ml) or anti-GFP antibody (5μg/ml) , respectively for 1h at room temperature followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-mouse IgG (1:10,000 dilution) at room temperature for 1 h. The blots were developed using ECL chemiluminescence reagent (Amersham Life Science, England) according to the manufacturers instructions.

Cloning, Expression and Purification of CD44 Cytoplasmic Domain (CD44cyt) from E. coli: The procedure for preparing the fusion protein of CD44's cytoplasmic domain was the same as described previously (48). Specifically, the cytoplasmic domain of human CD44 (CD44cyt) was cloned into pFLAG-AST using the PCR-based cloning strategy. Using human CD44 cDNA as template, one PCR primer pair (left,

FLAG-EcoRI; right, FLAG-XbaI) was designed to amplify complete CD44 cytoplasmic domain. The amplified DNA fragments were one-step cloned into a pCR2.1 vector and sequenced. Then, the DNA fragments were cut out by double digestion with EcoRI and XbaI and subcloned into EcoRI/XbaI double-digested pFLAG-AST (Eastman Kodak Co.-IBI, Rochester, NY) to generate FLAG-pCD44cyt construct. The nucleotide sequence of FLAG/CD44cyt junction was confirmed by sequencing. The recombinant plasmids were transformed to BL21-DE3 to produce FLAG-CD44cyt fusion protein. The FLAG-CD44cyt fusion protein was further purified by anti-FLAG M2 affinity gel column (Eastman Kodak Co.-IBI, Rochester, NY). The nucleotide sequence of primers used in this cloning protocol are:

FLAG-EcoRI: 5'-GAGAATTCGAACAGTCGAAGAAGGTGTCTCTTAAGC-3';

FLAG-XbaI: 5'-AGCTCTAGATTACACCCCAATCTTCAT-3'.

Expression Constructs: Both the full-length mouse Tiam1cDNA (FL1591) and the NH_2-terminally truncated Tiam1cDNA (C1199) were kindly provided by Dr. John G. Collard (The Netherlands Cancer Institute, The Netherlands). Specifically, the full-length Taim1 (FL1591) cDNA was cloned into the eukaryotic expression vector, pMT2SM. The NH_2-terminally truncated C1199 Tiam1 cDNA [carrying a hemagglutinin epitope (HA)-tag at the 3' end] was cloned into the eukaryotic expression vector, pUTSV1 (Eurogentec, Belgium). The Tiam1 fragment, PHn-CC-Ex domain was cloned into Calmodulin-Binding-Peptide (CBP)-tagged vector (pCAL-n)(Stratagen) using the PCR-based cloning strategy. Using human Tiam1 cDNA as a template, PHn-CC-Ex domain was amplified by PCR with two specific primers (left, 5'-AACTCGAGATGAGTACCACCAACAGTGAG-3' and right, 5'-AAAAAGCTTTCAGCCATCTGGAACAGTGTCATC-3') linked with specific enzyme digestion site (XhoI or Hind III). PCR product digested with Xho I and Hind III was purified with QIAquick PCR Purification Kit(Qiagen). The PHn-CC-Ex domain cDNA fragment was cloned into pCAL-n vector digested with Xho I and Hind III. The inserted PHn-CC-Ex domain sequence was confirmed by nucleotide sequencing analyses. The recombinant plasmids were transformed to BL21-DE3 to produce CBP-tagged PHn-CC-Ex fusion protein. This fusion protein was purified from bacteria lysate by calmodulin affinity resin column (Sigma).

The PHn-CC-Ex domain cDNA fragment was also cloned into pEGFPN1 vector (Clontech) digested with Xho I and Hind III to create GFP-tagged PHn-CC-Ex cDNA. The inserted PHn-CC-Ex domain sequence was confirmed by nucleotide sequencing analyses. This GFP-tagged PHn-CC-Ex domain cDNA was then used for a transient expression in SP1 cells as described below. The GFP tagged PHn-CC-Ex (M.W. ≈68kDa) is expressed as a 68kDa in SP1 or COS-7 cells by SDS-PAGE and immunoblot analyses.

Cell Transfection: To establish a transient expression system, SP1 cells (or COS-7 cells) were transfected with various plasmid DNAs (e.g. HA-tagged C1199 Tiam1cDNA, GFP-tagged PHn-CC-ExcDNA or HA-tagged C1199Tiam1cDNA plus GFP-tagged PHn-CC-ExcDNA (as co-transfection) or vector alone] using electroporation methods according to those procedures described previously (Chu et al., 1987). Briefly, SP1 cells were plated at a density of 2×10^6 cells per 100 mm dish and transfected with 25 μg/dish plasmid cDNA using electroporation at 230 v and 960 μFD with a Gene Pulser (Bio-Rad). Transfected cells were grown in the culture medium for at least 24-48 h. Various transfectants were then analyzed for their protein expression (e.g. Tiam1-related proteins) by immunoblot, GDP/GTP exchange reaction on Rac1, and tumor cell migration assays as described below.

In Vitro Binding of Tiam1/Tiam1 Fragment To CD44: Aliquots (0.5-1 ng protein) of purified FLAG-CD44cyt fusion protein bound to Anti-FLAG M2 antibody immunoaffinity beads were incubated in 0.5 ml of binding buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Triton X-100] containing various concentrations (10-800 ng/ml) of ^{125}I-labeled intact Tima1 (purified from SP1 cells) (5,000 cpm/ng protein) or ^{125}I-labeled recombinant Tiam1 fragment (CBP-tagged PHn-CC-Ex) at 4°C for 4 h. Non-specific binding was determined in the presence of a 50-100 fold excess of unlabeled Taim1 or CBP-PHn-CC-Ex in the presence of the same concentration of ^{125}I-labeled Tiam1 or ^{125}I-labeled CBP-PHn-CC-Ex Following binding, the immunobeads were washed extensively

in binding buffer and the beads-bound radioactivity was counted. Non-specific binding was approximately 20% of the total binding.

In some cases, 0.1 μg of FLAG-CD44cyt [biotinylated with EZ-link Biotin-LC-Hydrazide (Pierce Co., Rockford, IL)] or surface biotinylated CD44v3 was incubated with various Tiam1-related proteins (e.g. purified intace Tiam1, HA-tagged C1199, CBP-PHn-CC-Ex or CBP-coated beads) in the presence and absence of 100-fold excess amount of CBP-PHn-CC-Ex at room temperature in the binding buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Triton X-100] for 1 hr. After binding, biotinylated FLAG-CD44cyt or CD44v3 bound to the beads was analyzed by SDS-PAGE, transferred to the nitrocellulose filters and incubated with ExtrAvidin-peroxidase (Sigma Co.). After an addition of peroxidase substrate (Pierce Co.), the blots were developed using Renaissance chemiluminescence reagent (Amersham Life Science, England) according to the manufacturers instructions.

Tiam1-Mediated GDP/GTP Exchange For Rac1 Proteins: Purified E. coli-derived GST-tagged Rac1 (20pmole) was preloaded with GDP (30μM) in 10μl buffer containing 25mM Tris-HCl (pH 8.0), 1mM DTT, 4.7mM EDTA, 0.16mM MgCl2 and 200μg/ml BSA at 37° for 7min. In order to terminate preloading procedures, additional MgCl2 was then added to the solution (reaching a final concentration of 9.16mM) as described previously (40). Subsequently,2 pmole of Tiam1 (in anti-Tiam1-Sepahrose bead-conjugated forms) [isolated from COS-7 cells (transfected with either the full-lengh Tiam1cDNA or N-terminally truncated Tiam1cDNA) or SP1 cells (transfected with either the N-terminally truncated Tiam1cDNA or PHn-CC-ExcDNA) grown in the presence or absence of hyaluronic acid (100μg/ml)] or control samples (non-specific cellular material associated with preimmune serum-conjugated Sepharose beads) was preincubated with 0.25μM GTPγ^{35}S (1,250Ci/mmol) and 2.25μM GTPγS for 10 min followed by adding GDP-loaded GST-tagged Rac1GTPase. The amount of GTPγ^{35}S bound to Tiam1 (conjugated to anti-Tiam1-Sepharose beads) or control sample (preimmune serum-conjugated Sepharose beads) in the absence of Rac1GTPase was substracted from the original values. Data represent an average of triplicates from 3-5 experiments. The standard deviation was less than 5%.

Cell Adhesion Assay: SP1 cells were metabolically labeled with Tran^{35}S-label (20 μCi/ml) as described above. After labeling, the cells were washed in PBS and incubated in PBS containing 5 mM EDTA at 37° C to obtain a non-adherent single cell suspension. Labeled cells (≈ 9.1 x 10^5 cpm/10^5 cells) (in the presence or absence of anti-CD44v3 antibody) were plated on the hyaluronic acid (HA)-coated plates at 4° C for 30 min. Following incubation, the wells were washed three times in PBS, the adherent cells were solubilized in PBS containing 1% SDS and the well-bound radioactivity was determined by liquid scintillation counting. Non-specific binding was determined by including 300 μg/ml soluble HA during the incubation of cells on HA-coated wells. The non-specific binding was 10-15% of the total well-associated radioactivity and has been subtracted.

Tumor Cell Migration Assays: Twenty-four transwell units were used for monitoring *in vitro* cell migration as described previously (23,49). Specifically, the 8 μm porosity polycarbonate filters (CoStar Corp., Cambridge, MA) were used for the cell migration assay. SP1 cells [≈1 x 10^4 cells/well in phosphate buffered saline (PBS), pH 7.2] [in the presence or absence of anti-CD44v3 antibody (50 μg/ml)] were placed in the upper chamber of the transwell unit. In some cases, cells were transfected with either C1199Tiam1cDNA, PHn-CC-ExcDNA, C1199Tiam1cDNA plus PHn-CC-ExcDNA or vector alone. The growth medium containing high glucose DMEM supplemented with 200 μg/ml hyaluronic acid was placed in the lower chamber of the transwell unit. After 18 h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, vital stain MTT (Sigma Co., St. Louis, MO) was added at a final concentration of 0.2 mg/ml to both the upper and the lower chambers and incubated for additional 4 hours at 37°C. Migrative cells at the lower part of the filter were removed by swabbing with small pieces of Whatman filter paper. Both the polycarbonate filter and the Whatman paper were placed in dimethylsulfoxide to solubilize the crystal. Color intensity was measured in 450 nm. Cell migration processes were determined by measuring the

cells that migrate to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (23,49). The CD44-specific cell migration was determined by subtracting non-specific cell migration (i.e. cells migrate to the lower chamber in the presence of anti-CD44v3 antibody treatment) from the total migrative cells in the lower chamber. The CD44-specific cell migration in vector-transfected cells (control) is designated as 100%. Each assay was set up in triplicate and repeated at least 3 times. All data were analyzed statistically using the Student's t test and statistical significance was set at $p<0.01$.

RESULTS

Identification of CD44 Variant Isoform(s) as Hyaluronic Acid (HA) Receptor(s) in SP1 Cells:

The expression of CD44 variant isoforms such as CD44v3 is known to be closely correlated with metastatic and proliferative behavior of a variety of tumor cells including various carcinomas such as human breast tumor cells (14-19). Immunoblotting with anti-CD44v3 antibody [recognizing the v3-specific sequence located at the membrane proximal region of CD44's extracellular domain] indicates that a single CD44v3 protein (M. W. ≈ 260kDa) is expressed in SP1 cells (Fig. 1, lane 1). Furthermore, we have utilized surface biotinylation techniques and anti-CD44v3-mediated immunoprecipitation to characterize this CD44v3 molecule. Our results show that the 260kDa CD44v3 molecule can be surface-biotinylated and is located on the surface of SP1 cells (Fig. 1, lane 2). No $CD44v_3$-containing material is observed in control samples when preimmune rabbit serum is used in either immunoblot (Fig. 1, lane 3) or immunoprecipitation experiments (Fig. 1, lane 4). Further analyses using RT-PCR, cloning and nucleotide sequence techniques indicates that this CD44v3 belongs to the $CD44v_{3,8-10}$ isoform in SP1 cells (data not shown). This $CD44v_{3,8-10}$ variant exon structure was previously identified in human breast carcinoma samples (14-19) and its molecular mass (expressed at the protein level) has been shown to be ≈260kDa (9).

CD44 is the major hyaluronan cell surface receptor (50), and a cellular adhesion molecule in many different cell types (51). Specific hyaluronic acid (HA) binding motifs have been identified and localized in the extracellular domain of all CD44 isoforms (52,53). To determine whether HA promote cell adhesion, breast tumor cells (SP1 cell line) were incubated with plastic dishes coated with hyaluronic acid (HA). As shown in Table 1, SP1 cells adhere to the HA-coated dishes very well. In addition, since preincubation with anti-CD44v3 antibody blocks the adhesion of SP1 cells to HA-coated dishes, these data clearly indicates that CD44v3 isoform involves a specific binding interaction with the extracellular matrix component (ECM) such as HA and is a cell surface adhesion molecule in SP1 cells.

Analysis of A Complex Formed Between CD44v3 and Tiam1 SP1 Cells in vivo:

Both CD44v isoforms (14-19) and Tiam1 (39) have been detected in a variety of tumor cells. In this study we have addressed the question of whether there is an interaction between CD44v3 isoform and Tiam1 in breast tumor cells (e.g. SP1 cells). First, we have analyzed Tiam1 expression (at the protein level) in breast tumor cells such as SP-1 cell line. Immunoblot analysis, utilizing anti-Tiam1 antibody designed to recognize the specific epitope located at the C-terminal sequence of Tiam1 reveals a single polypeptide (M. W. ≈ 200kDa) (Fig. 2, lane 2). We have demonstrated that Tiam1 detected in SP1 cells revealed by anti-Tiam1-mediated immunoblot is specific since no protein is detected in these cells using preimmune rabbit IgG (Fig. 2A, lane 1). Furthermore, we have carried out anti-CD44v3-mediated and anti-Tiam1-mediated precipitation followed by anti-Tiam1 immunoblot or anti-anti-CD44v3 immunoblot, respectively, using SDS-PAGE analyses. Our results clearly indicate that the Tiam1 band is revealed in anti-CD44v3-immunoprecipitated materials (Fig. 2, lane 3). The CD44v3 band can also be detected in the anti-Tiam1-immunoprecipitated materials (Fig. 2, lane 4).

These findings clearly establish the fact that CD44v3 and Tiam1 are closely associated with each other in vivo in breast tumor cells.

In vitro Binding Between E. coli-Derived CBP-PHn-CC-Ex of Tiam1 and E. coli-Derived FLAG-CD44cyt:

Previous studies indicate that Tiam1's membrane localization [through its NH2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain (designated as PHn-CC-Ex)] (Fig. 3A and C) is required for the activation of Rac1 signaling pathways leading to membrane ruffling and C-Jun NH2-terminal kinase activation (37,54). To test whether CD44 is one of the membrane proteins involved in the direct binding to Tiam1, we have used the calmodulin-binding protein (CBP)-tagged PHn-CC-Ex fusion protein (Fig. 3C; Fig 4, lane 1) and the FLAG-tagged cytoplasmic domain of CD44 (FLAG-CD44cyt) fusion protein (Fig. 4, lane 2) to identify the CD44 binding site on the Tiam1 molecule. Specifically, biotinylated FLAG-CD44cyt was incubated with CBP-PHn-CC-Ex-coated beads or CBP-coated beads alone in vitro. Our results indicate that a significant amount of the biotinylated FLAG-CD44cyt fusion protein becomes associated with the CBP-PHn-CC-Ex beads (Fig. 5, lane 1) but not with CBP-beads only (Fig. 5, lane 2). Furthermore, we have tested the binding of FLAG-CD44cyt to ^{125}I-labeled CBP-PHn-CC-EX (or ^{125}I-labeled intact Tiam1) under equilibrium binding conditions. Scatchard plot analyses presented in Fig. 6 indicate that PHn-CC-Ex binds to the cytoplasmic domain of CD44 (CD44cyt) at a single site (Fig. 6A) with high affinity [an apparent dissociation constant (K_d) of ≈ 1.0 nM]. This interaction between PHn-CC-Ex and CD44 is comparable in affinity to CD44 binding (Kd ≈2.0 nM) to intact Tiam1 (Fig. 6B). These findings clearly indicate that Tiam1 (in particular, PHn-CC-Ex domain) contains the CD44 binding site.

Tiam1 Acts As An Upstream Activator For Rac1 in SP1 Cells

Rac1 GTPase becomes activated when bound GDP is exchanged for GTP by a process catalysed by guanine nucleotide (GDP-GTP) exchange factors (GEFs) or GDP-dissociation stimulator (GDS) proteins [i.e. promoting GTP binding to RhoA by facilitating the release of GDP] (25,26). Tiam1 is known to function as an exchange factor for the Rho-like GTPases such as Rac1 (34,40-42). To investigate whether Tiam1 complexed with CD44v3 acts as a GDP/GTP exchange factor [or a GDP-dissociation stimulator (GDS) protein] for E. coli-derived GST-Rac1, we have isolated Tiam1 complexed with CD44v3 from SP1 cells using anti-Tiam1-conjugated Sepharose beads. Our data show that Tiam1 complexed with CD44v3 from SP1 cells causes the exchange of preloaded GDP for [^{35}S]GTPγS on GST-Rac1 in a time-dependent manner (Fig. 7a and 7b). Most importantly, addition of HA to $CD44v_3$ containing SP1 cells stimulates the total amount of bound [^{35}S]GTPγS to GST-Rac1 (Fig. 7b) (at least 1.5-fold increase) as compared with Tiam1 isolated from untreated SP1 cells (Fig. 7b) or HA-treated SP1 cells in the presence of anti-CD44v3 antibody (data not shown). No [^{35}S]GTPγS-bound material was detected in these samples containing GST alone under the same GDP/GTP exchange reaction using Tiam1 isolated from SP1 cells [in the presence (Fig. 7c) or absence (Fig. 7d) of HA treatment]. These findings suggest that the HA interaction with $CD44v_3$ isoform-containing SP1 cells promotes Tiam1 activation of Rac1.

Effect of Tiam1 On HA-Mediated Tumor Cell Migration

Previous studies indicate that the invasive phenotype of tumor cells characterized by an "invadopodia" structure (or membranous projections) (56,57) and tumor cell migration (28,29) is closely associated with $CD44v_{3,8-10}$-linked cytoskeleton function (23). In this study we have transiently transfected breast tumor cells (e.g. SP-1 cells) with GFP-tagged PHn-CC-Ex Tiam1 cDNA (Fig. 3C) or HA-tagged N-terminally truncated C1199 Tiam1 cDNA (Fig. 3B). Our results show that the PHn-CC-Ex domain and C1199 Tiam1 are expressed as a 68 kDa protein (Fig. 8, lane 1) and a 150 kDa protein (Fig. 8, lane 2), respectively, using anti-GFP-mediated or anti-HA-mediated immunoblot in $CD44v_3$-positive breast tumor cells (SP1 cells). No protein band was detected in vector-transfected SP1 cells by anti-GFP or anti-HA immunoblot (data not shown).

Further analyses using an in vitro binding assay show that surface biotinylated CD44v3 (isolated from SP1) specifically binds to Tiam1 [including intact Tiam1 (Fig. 9, lane 1), HA-tagged C1199 Tiam1 (Fig. 9, lane 2) or Tiam1 fragment (PHn-CC-Ex) (Fig. 9, lane 3)]-coated beads. in the presence of an excess amount (≈100-fold) of recombinant PHn-CC-Ex Tiam1 fragment, the binding interaction between CD44v3 and theseTiam1-related proteins is readily abolished (Fig. 9, lane 4-6). These observations suggest that Tiam1 fragment such as PHn-CC-Ex acts as a potent competitive inhibitor for Tiam1 association with CD44v3 in vitro.

Furthermore, we have demonstrated that overexpression of C1199 Tiam1 (by transfecting Met-1 cells with C1199 Tiam1 cDNA) promotes C1199 Tiam1 association with CD44v3 (Table 2A), stimulates the total amount of GDP/GTP exchange on Rac1 (Table 2B), and induces a significant amount of increase in CD44v3-specific and HA-mediated breast tumor cell migration (Table 2C) compared to PHn-CC-Ex cDNA-transfected or vector-transfected SP1 transfectants (Table 2A-C). Treatment of SP1 cells (e.g. untransfected cells or various transfectants) with certain agents [e.g. cytochalasin D (a microfilament inhibitor)] causes a remarkable inhibition of tumor cell migration (Table 2C). In addition, we have co-transfected SP1 cells with PHn-CC-Ex cDNA and C1199 Tiam1 cDNA. Our results indicate that both PHn-CC-Ex and C1199 Tiam1 are co-expressed as a 68kDa protein and a 150kDa, respectively, in SP1 cells (Fig. 8, lane 3). Most importantly, we have found that PHn-CC-Ex not only effectively inhibits C1199 Tiam1 association with CD44v3 (Table 2A) and Rac1 activation (Table 2B) but also efficiently blocks C1199 Tiam1-activated CD44v3-dependent and HA-mediated breast tumor cell migration (Table 2B). These results are consistent with previous report that co-transfection of COS-7 cells with PHn-CC-Ex cDNA and C1199 Tiam1 cDNA results in an inhibition of C1199 Tiam1-induced membrane ruffling (54). These findings suggest that the NH2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain (PHn-CC-Ex) are involved in Tiam1 localization to the plasma membrane proteins such as CD44v3 isoforms, and for oncogenic signaling during extracellular matrix component (e.g. hyaluronic acid)-regulated breast tumor cell invasion and migration.

(7) KEY RESEARCH ACCOMPLISHMENTS

• We have found that Tiam1 (T lymphoma invasion and metastasis), one of the known guanine nucleotide (GDP/GTP) exchange factors (GEFs) for Rac1GTPases, is expressed in breast tumor cells (SP1 cell line).
•Immunoblot and immunoprecipitation analyses indicate that both the Tiam1 protein and the metastasis-specific surface molecule, CD44v3 [the hyaluronic acid (HA) binding receptor] are expressed in breast tumor cells, and that these two proteins are physically associated as a complex in vivo.
• Using an E. coli-derived calmodulin-binding peptide (CBP)-tagged Tiam1 fragment [i.e. the NH2-terminal pleckstrin homology (PHn) domain and an adjacent protein interaction domain (designated as PHn-CC-Ex, aa393-aa738 of Tiam1] and an in vitro binding assay, we have detected a specific binding interaction between the Tiam1's PHn-CC-Ex domain and CD44.
• Scatchard plot analysis indicates that there is a single high affinity CD44 binding site in Tiam1's PHn-CC-Ex domain with an apparent dissociation constant (Kd) of 1nM which is comparable to CD44 binding (Kd ≈2nM) to intact Tiam1. These findings suggest that the PHn-CC-Ex domain is the primary Tiam1 binding region for CD44.
• Most importantly, the binding of HA to CD44v3 of SP1 cells stimulates Tiam1-catalyzed Rac1 signaling and cytoskeleton-mediated tumor cell migration.
• Transfection of SP1cells with Tiam1cDNA promotes Tiam1 association with CD44v3 and upregulates Rac1 signaling as well as HA/CD44v3-mediated breast tumor cell migration.
• Co-transfection of SP1 cells with PHn-CC-Ex cDNA and Taim1 cDNA effectively inhibits Tiam1association with CD44 and efficiently blocks tumor behaviors. Taken together, we believe that the linkage between CD44v3 isoform and the PHn-CC-EX domain of Tiam1 is required for HA stimulated Rac1 signaling and cytoskeleton-mediated tumor cell migration during breast cancer progression.

(8) **REPORTABLE OUTCOMES**

-Manuscript: Bourguignon, L.Y.W., H. Zhu, L. Shao and Y.W. Chen, Ankyrin interaction with Tiam1 regulates Rac1 signaling and $CD44v_{3,8-10}$-associated metastatic breast tumor cell invasion and migration (to be submitted to J. Cell Biology, 1999).
-Manuscript: Bourguignon, Lilly Y.W., H. Zhu, L. Shao, B. Elliott and Y.W. Chen. CD44 interaction with Tiam1 protmotes Rac1 signaling and hyaluronic acid (HA)-mediated breast tumor cell migration. (to be submitted to J. Biol. Chem, 1999).
-Abstract: Bourguignon, L.Y.W., H.Zhu, L. Shao, and Y.W.Chen, Identification Of An Ankyrin-Binding Domain In TIAM1 And Its Role In Regulating $CD44v_{3,8-10}$-Associated Metastatic Breast Tumor Cell Invasion And Migration. Proc. Am. Assoc. Cancer Res. 40:196 (1999).
-Presentations: This work has been presented in the Minisymposium (Cell and Tumor Biology 7-Cellular Signaling in Cancer Invasion and Metastasis) of the American Association for Cancer Research Meeting at Philadelphia, PA in 1999.

(9) CONCLUSIONS

CD44 denotes a family of glycoproteins [e.g. CD44s (standard form), CD44E (epithelial form) and CD44v (variant isoforms)] which are expressed in a variety of cells and tissues (1-6). Clinical studies indicate that a number of CD44v isoforms have been detected at high levels on the surface of tumor cells during tumorigenesis and metastasis (13-17). As the histologic grade of each of the tumors progresses, the percentage of lesions expressing an associated CD44v isoform increases. In particular, the CD44v3-containing isoforms are detected preferentially on highly malignant breast carcinoma tissue samples. In fact, there is a direct correlation between CD44v3 isoform expression and increased histologic grade of the malignancy (14,17,57).

It has been speculated that some of these CD44v3 isoforms on epithelial cells may act as surface modulators to facilitate unwanted growth factor receptor-growth factor interactions (9,10) and subsequent tumor formation. The CD44-related glycoproteins are also known to mediate cell adhesion to extracellular matrix (ECM) components [e.g. hyaluronic acid (HA)] and to function as the major hyaluronate receptor (50). In this study we have demonstrated that a 260kDa CD44v3 isoform is expressed on the surface of breast tumor cells (SP1 cell line) (Fig. 1) and that it interacts with extracellular matrix, hyaluronic acid (HA) as an adhesion receptor (Table 1). Furthermore, addition of HA to SP1 cells stimulates tumor cell migration in a CD44v3-specific and cytoskeleton-dependent manner (Table 2). These findings are consistent with previous findings that CD44v isoforms expressed in tumor cells often display enhanced hyaluronic acid binding which increases cell migration capability (58,59).

The invasive phenotype of CD44v3-mediated breast tumor cells, characterized by "invadopodia" formation (23), MMP-9 activation (23,24) and tumor cell motility (23,48), has been linked to cytoskeletal function, a process in which the small GTP-binding proteins such as RhoA and Rac1 are shown to play important roles. Tsukita and his co-workers have reported that Rho-like proteins participate in the interaction between the CD44 and the ERM cytoskeletal proteins (60). Our recent study determined that RhoA is physically linked to CD44v3 isoform (e.g. $CD44v_{3,8-10}$) in breast tumor cells (48). Rho-kinase (ROK) stimulated by activated RhoA (GTP-bound form of RhoA) appears to play a pivotal role in promoting $CD44v_{3,8-10}$-ankyrin interaction during membrane-cytoskeleton function and metastatic breast tumor cell migration (48). Signaling to the RacGTPase known to regulate actin assembly associated with membrane ruffling, pseudopod extension, cell motility and cell transformation (33-37) has been shown to be abnormal in breast tumor cells as compared to normal breast epithelial cells (61). The fact that Rac1 induces stress fiber formation in a Rho-dependent manner suggests that "cross-talk" occurs between the Rho and Rac1 signaling pathways (33). The question of whether the activation of Rac1 signaling is involved in CD44v3-cytoskeleton-mediated breast tumor-specific events remains to be answered.

Tiam1, which was identified by retroviral insertional mutagenesis and selected for its invasive cell behavior in vitro, has been shown to regulate Rac1 activation (38,39). This molecule is largely hydrophilic and contains several functional domains including a Dbl homology (DH) domain (38,62,63), a Discs-large homology region (DHR) (38,64), and two pleckstrin homology (PH) domains [e.g. PHn (the PH domain located at the NH2-terminal region of the molecule; and PHc (the PH domain located at the COOH-terminal region of the molecule)] (Fig. 3) (38). In particular, the DH domain of Tiam1 exhibits GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (62,63) and plays an important role in Rac1 signaling and cellular transformation (33-37). In breast tumor cells (e.g. SP1 cells), Tiam1 is detected as a 200 kD protein (Fig. 2) that is capable of carrying out GDP/GTP exchange for Rac1 (Fig. 7), similar to Tiam1 described in other cell types (34, 40-42,65,66). Other functional domains such as DHR have been implicated in the binding of membrane protein networks (38,64). The pleckstrin homology (PH) domain may mediate association

with the submembrane region of the cell via protein-protein or protein-lipid interactions (67). Based on mutational analyses and immunofluorescence staining, Collard and co-workers report that the NH2-terminal pleckstrin homology (PHn) domain (but not PHc) and an adjacent protein interaction domain (e.g. PHn-CC-Ex domain) (Fig. 3) are required for Tiam1 targeting to the plasma membrane and Rac1 activation in fibroblasts (37,54). At the present time, identification of the membrane protein(s) involved in Tiam1 binding has not been established.

In this study we have presented new evidence that a close interaction occurs between Tiam1 and certain plasma membrane proteins such as CD44v3 isoform. Using two recombinant proteins [CBP-tagged PHn-CC-Ex domain (Fig. 4, lane 1) and FLAG-tagged CD44 cytoplasmic domain (FLAG-CD44cyt) (Fig. 4, lane 2)], we have demonstrated that the PHn-CC-Ex domain of Tiam1 is directly involved in the binding to the cytoplasmic domain of CD44 (Figs. 5 and 6). In fact, the binding affinity of Tiam1's PHn-CC-Ex domain to CD44 is slightly higher than that of intact Tiam1 binding to CD44 (Figs. 5 and 6). In the presence of PHn-CC-Ex , the binding between Tiam1 and CD44 (e.g. CD44v3) is greatly reduced (Fig. 9). The ability of PHn-CC-Ex to effectively compete for Tiam1 binding to the plasma membrane proteins such as CD44v3 strongly suggests that Tiam1's PHn-CC-Ex is responsible for the recognition of CD44 in vitro.

In addition, we have detected that Taim1 and CD44v3 are physically linked to each other as a complex in vivo (Fig. 2 and Table 2A) and that HA binding to CD44v3 promotes Tiam1-catalyzed Rac1 activation (Table 2B) and tumor cell migration (Table 2C). These results suggest that Tiam1 and CD44v3 are not only structurally linked but also functionally coupled. Furthermore, our data indicate that overexpression of Tiam1 [by transfecting SP1 cells with C1199 Tiam1cDNA] (Fig. 8) promotes Tiam1 association with CD44v3 (Table 2) and metastatic breast tumor behaviors (e.g. Rac1 activation and tumor cell migration) (Table 2). Co-transfection of SP1 cells with PHn-CC-Ex cDNA and C1199 Tiam1cDNA (Fig. 8) effectively blocks C1199 Tiam1-activated and HA-mediated/CD44v3-specific tumor cell-specific behaviors (e.g. Tiam1 association with CD44v3, Rac1 signaling, tumor cell growth and migration) (Table 2) . These findings further support our conclusion that PHn-CC-Ex domain of Tiam1 interacts with CD44v3 in vivo (Table 2). Recently, we have also identified a unique sequence residing within the PHn-CC-Ex domain as the putative cytoskeletal binding site of Tiam1 (68). Most importantly, interaction between Tiam1 and the cytoskeleton up-regulates the GDP/GTP exchange activity of Rho-like GTPases and stimulates breast tumor cell invasion/migration (68). These observations clearly support the notion that the PHn-CC-Ex fragment of Tiam1 is one of the important regulatory domains required for Tiam1 function.

In fibroblasts, Tiam1-induced membrane ruffling is dependent on Rac1 (but not RhoA) activity (69). The fact that Tiam1 is involved in both Rac1- and RhoA-mediated pathways during neurite formation in nerve cells suggests that the balance between two Tiam1-activated Rho-like GTPases determines a particular biological activity (65). Tiam1-Rac1 signaling is also implicated in promoting integrin-mediated cell-cell and cell-extracellular matrix interaction and lymphoid cell invasion (34,65). In addition, the laminin receptor, $\alpha 6\beta 1$ integrin appears to require Rac1 as a downstream of Tiam1 signaling in neuroblastoma cell activation (65). In epithelial Madin-Darby canine kidney (MDCK) cells, fibronectin and/or laminin1-induced Tiam1-Rac1 signaling up-regulates E-cadherin-mediated adhesion and plays an invasion-suppressor role in Ras-transformed MDCK cells (66). However, if MDCK cells were grown on different collagen substrates, the expression of Tiam1 or constitutively activated Rac1 (V12Rac) in these cells is able to inhibit the appearance of E-cadherin adhesion and promote cell migration (70,71). Apparently, various responses by Tiam1-catalyzed Rho-like GTPases are controlled by specific upstream activators [in particular, cell adhesion receptors (e.g. CD44, integrin or E-cadherin, etc.) or extracellular matrix components (e.g. HA, collagens, laminin or fibronectin, etc.)] which may result in selective Tiam1-activated Rho-like GTPases and

distinct biological outcome. In summary, we believe that Tiam1-CD44v3 interaction plays a pivotal role in regulating oncogenic signaling required for RhoGTPase activation and cytoskeleton function during HA-mediated metastatic breast tumor cell progression. This could be one of the critical steps in CD44 variant isoform-mediated breast tumor spread and metastasis.

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(11) APPENDICES

Table 1: CD44v3-Mediated Adhesion of Metabolically Labeled SP1 Cells to Hyaluronic Acid (HA)-Coated Plates.

Treatments	CD44v3-Specific Adhesion To HA-Coated Plates	
	(CPM)	(% of Control)
Untreated cells (Control)	5,057±201	100%
Anti-CD44v3 treated cells	1,091±136	21%

Tran^{35}S-labeled SP1 cells were pretreated with or without anti-CD44v3 antibody treatment. Subsequently, these cells were incubated in tissue culture wells coated with hyaluronic acid (HA) as described in the Materials and Methods. The background level of binding was determined by cell adhesion performed in the presence of an excess amount of soluble HA. The results were expressed in terms of HA-specific binding in which the background levels of binding have been subtracted. Data are expressed as mean cpm ± SEM of triplicate determinations.

Table 2: Measurement of C1199 Tiam1-CD44v3 Association, Tiam1-Catalyzed Rac1 Activation and HA-Mediated/Cytoskeleton-Associated Breast Tumor Cell Migration.

A: Tiam1-CD44v3 Association:

Cells	C1199 Tiam1-CD44v3 Association[a] (% of Control)[b]
Untransfected cells (control)	100
Vector-transfected cells	99
PHn-CC-ExcDNA-transfected cells	85
C1199 Tiam1cDNA-transfected cells	150
PHn-CC-ExcDNA and C1199Tiam1cDNA co-transfected cells	89

B: Tiam1-Catalyzed Rac1 Activation:

Cells	Amount of [γ-^{35}S]GTP bound to GST-Rac1[a] (CPM)	(% of Control)[b]
Untransfected cells (control)	15,000	100
Vector-transfected cells	14,700	98
PHn-CC-ExcDNA-transfected cells	13,200	88
C1199 Tiam1cDNA-transfected cells	22,800	152
PHn-CC-ExcDNA and C1199Tiam1cDNA co-transfected cells	13,500	90

C: In vitro Cell Migration:

Cells	HA-Mediated/CD44v3-Specific Cell Migration[a] (% of Control)[b] DMSO-treated control	Cytochalasin D-treated
Untransfected cells (control)	100	20
Vector-transfected cells	97	25
PHn-CC-ExcDNA-transfected cells	85	18
C1199 Tiam1cDNA-transfected cells	155	50
PHn-CC-ExcDNA and C1199Tiam1cDNA co-transfected cells	90	15

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a: SP1 cells [$\approx$1 x 10^4 cells/well in phosphate buffered saline (PBS), pH 7.2] [in the presence or absence of cytochalasin D (dissolved in DMSO) (20μg/ml), or DMSO alone] were placed in the upper chamber of the transwell unit. In some cases, SP1 cells were transfected with either HA-tagged C1199 Tiam1 cDNA or GFP-tagged PHn-CC-ExcDNA or HA-tagged C1199 Tiam1cDNA plus GFP-tagged PHn-CC-ExcDNA, or vector alone. After 18h incubation at 37°C in a humidified 95% air/5% CO_2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swap. Cell migration processes were determined by measuring the cells that migrate to the lower side of the polycarbonate filters containing hyaluronic acid by standard cell number counting assays as described in the Materials and Methods. For detecting HA-tagged C1199 association with CD44v3 (in the absence or presence of GFP-tagged PHn-CC-Ex overexpression), NP-40 solubilized SP1 transfectants were immunoprecipited with anti-CD44v3 antibody followed by anti-HA or anti-GFP-mediated immunoblot as described in the Materials and methods. Procedures for measuring Tiam1-catalyzed GDP/GTP exchange reaction on GST-Rac1 were described in the Materials and Methods. Each assay was set up in triplicate and repeated at least 3 times. All data were analyzed statistically by Student's t test and statistical significance was set at $p<0.01$. In these experiments $\approx$30 to 40% of input cells ($\approx$1 x 10^4 cells/well) undergo in vitro cell invasion and migration in the control samples.

b: The values expressed in this table represent an average of triplicate determinations of 3-5 experiments with a standard deviation less than ±5%.

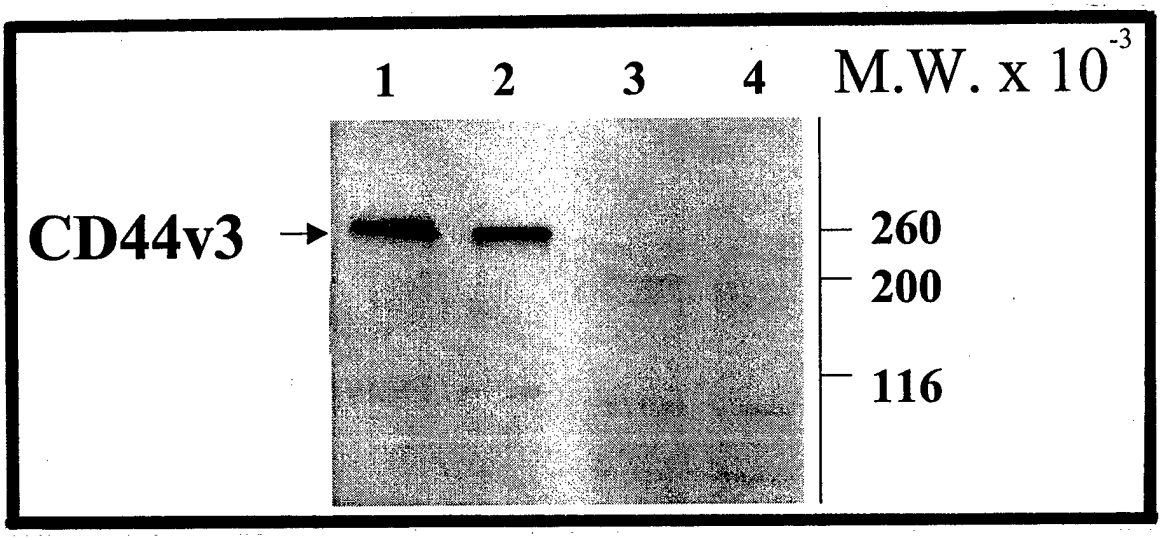

Fig. 1: Expression of CD44v3 in breast tumor cells.

Breast tumor cells (SP1 cell line) were surface biotinylated (or unlabeled) and solubilized in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 1% Triton X-100. The solubilized materials were then immunoprecipitated by anti-CD44v3 antibody as described in the Materials and Methods.

Lane 1: Immunblot of unlabeled SP1 cell lysate using rabbit anti-CD44v3 antibody.

Lane 2: Immunoprecipitation of surface biotinylated SP1 cells using rabbit anti-CD44v3 antibody.

Lane 3: Immuoblot of unlabeled SP1 cells with preimmune rabbit serum.

Lane 4: Immunoprecipitation of surface biotinylated SP1 cells with preimmune rabbit serum.

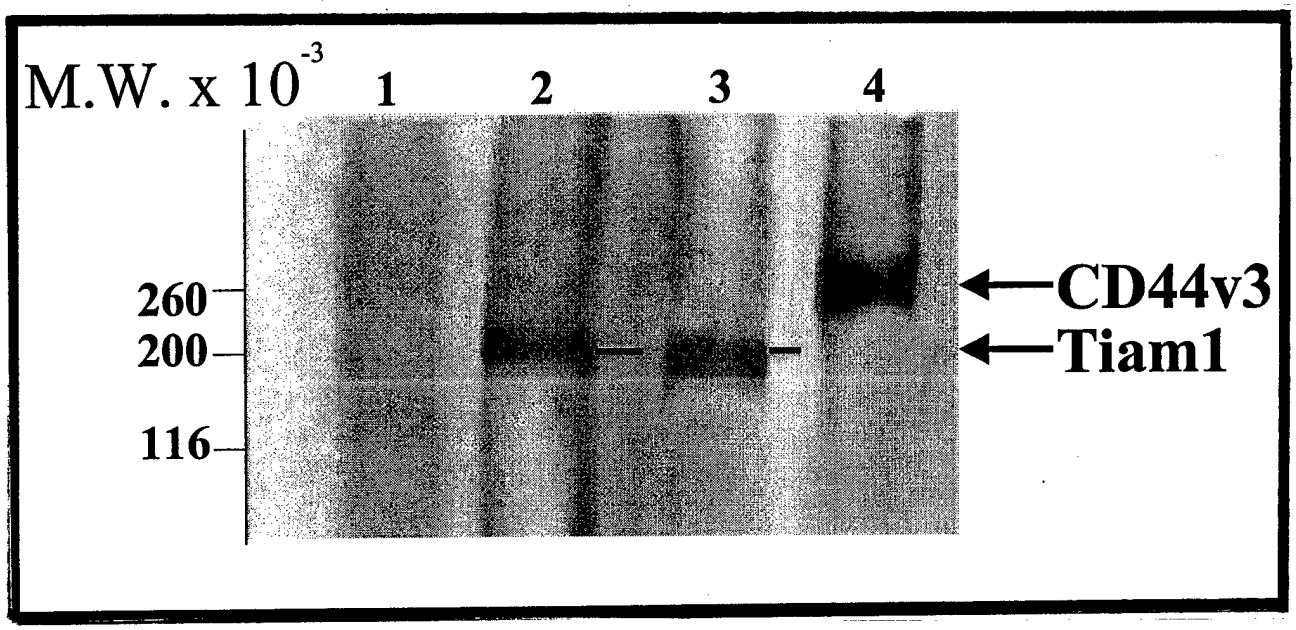

Fig. 2: Detection of Tiam1 and Tiam1-CD44v3 complex in SP1 cells.
SP1 cells (5 x 10^5 cells) were solubilized by 1% Nonidet P-40 (NP-40) buffer followed by immunoprecipitation and/or immunblot by anti-Tiam1 antibody or anti-CD44v3 antibody, respectively as described in the Materials and Methods.
Lane 1: Immunoblot of SP1 cells with preimmune rabbit serum.
Lane 2: Detection of Tiam1 with anti-Tiam1-mediated immunoblot of SP1 cells.
Lane 3: Detection of Tiam1 in the complex by anti-CD44v3-immunoprecipitation followed by immunoblotting with anti-Tiam1 antibody.
Lane 4: Detection of CD44v3 in the complex by anti-Tiam1 immunoprecipitation followed by immunoblotting with anti-CD44v3 antibody.

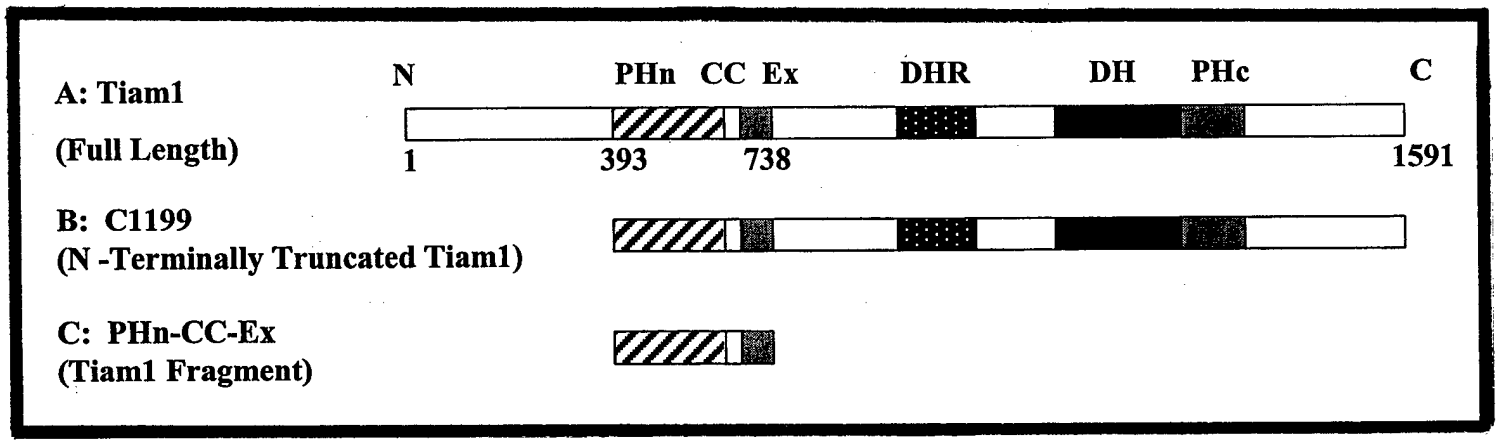

Fig. 3: Illustration of Tiam1 full-length (A) and deletion mutant cDNA constructs (B and C). The full length Tiam1 contains DH, *dbl*-homology domain; DHR, *discs-large* homology domain; two pleckstrin homology (PH) domains [including the NH2-terminal PH (PHn) and the COOH-terminal PH (PHc)]; CC, a putative coiled coil region; and Ex, an additional adjacent region. The N-terminally truncated C1199 Tiam1 encodes the C-terminal 1199 amino acids (B). PHn-CC-Ex domain of Tiam1 encodes the sequence between aa393-aa738 (C).

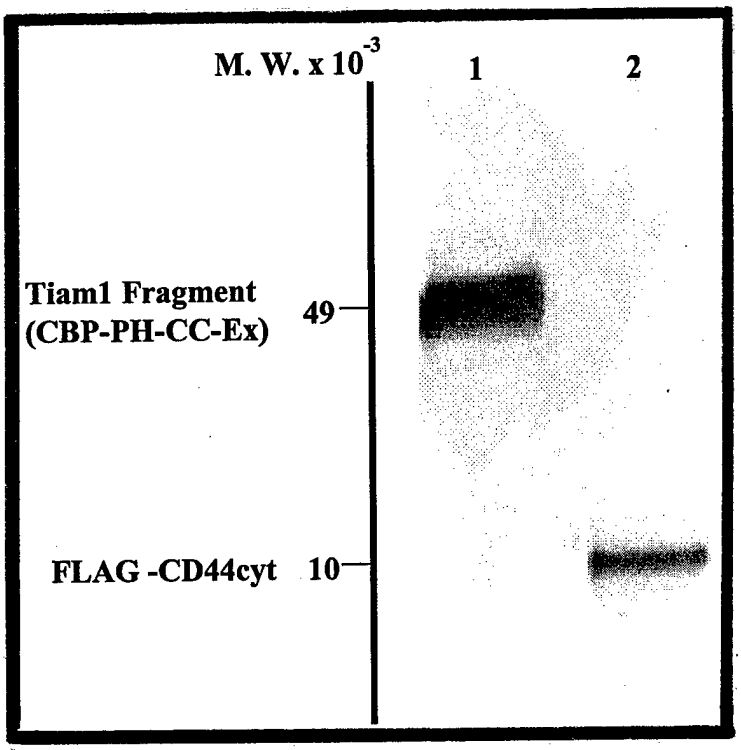

Fig. 4: Characterization of various recombinant proteins used in the <u>in</u> <u>vitro</u> binding assay.
Lane 1: A coomassie blue staining of CBP-PH-CC-Ex fusion protein purified by calmodulin affinity resin column chromatography.
Lane 2: A coomassie blue staining of FLAG-CD44cyt fusion protein eluted from affinity column chromatography with FLAG peptide.

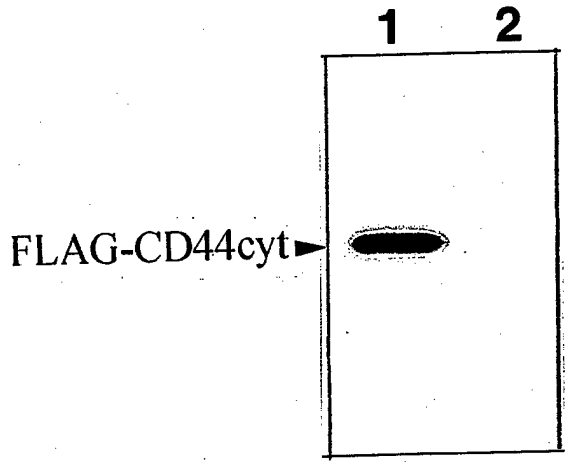

Fig. 5: Binding of FLAG-CD44cyt to CBP-PHn-CC-Ex-conjugated beads.
Lane 1: Association of biotinylated FLAG-CD44cyt with CBP-PHn-CC-Ex-conjugated beads.
Lane 2: Association of biotinylated FLAG-CD44cyt with CBP-conjugated beads.

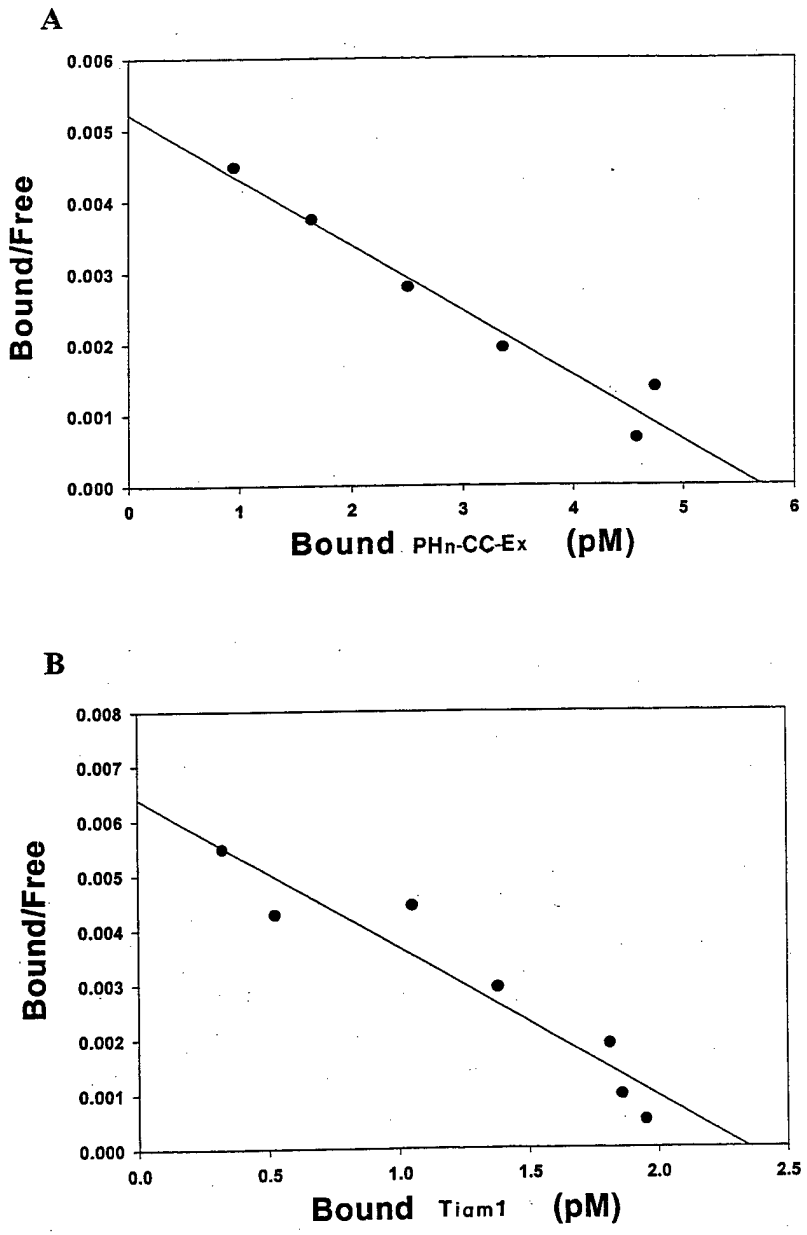

Fig. 6: Binding of ^{125}I-labeled PHn-CC-Ex (or Tiam1) to FLAG-CD44cyt.

Various concentrations of ^{125}I-labeled PHn-CC-Ex (or Tiam1) were incubated with FLAG-CD44cyt-coupled beads at 4°C for 4 h. Nonspecific binding was determined in the presence of 50-fold excess of unlabeled PHn-CC-Ex (or Tiam1) and subtracted from the total binding. Results represent an average of duplicate determinations from the same experiment. Data presented is the representative of three individual binding experiments.

A: Scatchard plot analysis of the equilibrium binding data between ^{125}I-labeled PHn-CC-Ex and FLAG-CD44cyt.

B: Scatchard plot analysis of the equilibrium binding data between ^{125}I-labeled intact Tiam1 and FLAG-CD44cyt.

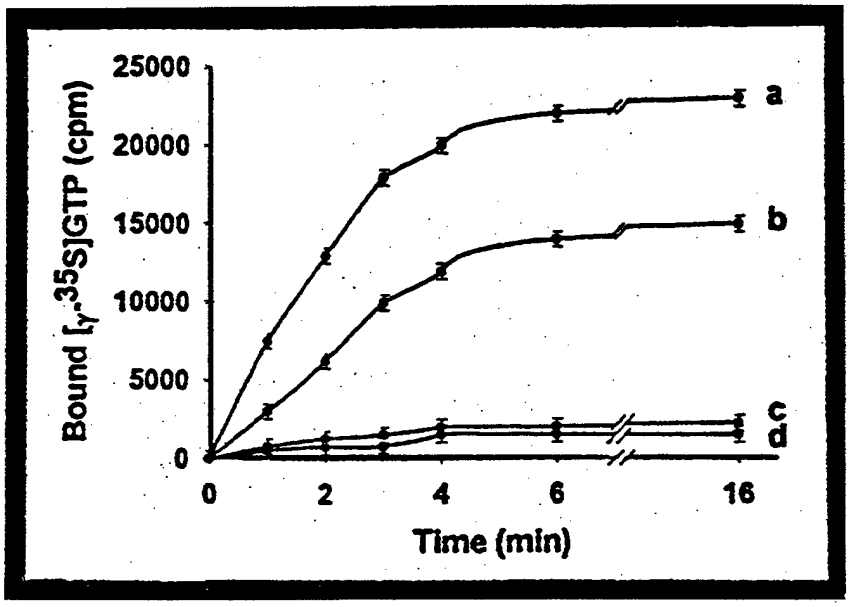

Fig. 7: Tiam1-mediated GDP/GTP exchange for Rac1 protein.

Tiam1 isolated from SP1 cells (treated with HA or without any treatment) was preincubated for 10 min with 0.25μM GTPγ^{35}S (1,250Ci/mmol) and 2.25μM GTPγS (or in the presence of 1mM unlabeled GTPγS) followed by adding GDP-loaded GST-Rac1 GTPases (or GST alone). The amount of GTPγ^{35}S bound to samples in the absence of GTPases was substracted from the original values. Data represent an average of triplicates from 3-5 experiments. The standard deviation was less than 5%.

a: Kinetics of GTPγ^{35}S bound to GDP-loaded GST-Rac1 in the presence of Tiam1 (isolated from SP1 cells treated with HA).

b:Kinetics of GTPγ^{35}S bound to GDP-loaded GST-Rac1 in the presence of Tiam1 (isolated from SP1 cells without any treatment).

c:Kinetics of GTPγ^{35}S bound to GDP-treated GST in the presence of Tiam1 (isolated from SP1 cells treated with HA).

d:Kinetics of GTPγ^{35}S bound to GDP treated GST in the presence of Tiam1 (isolated from SP1 cells without any treatment).

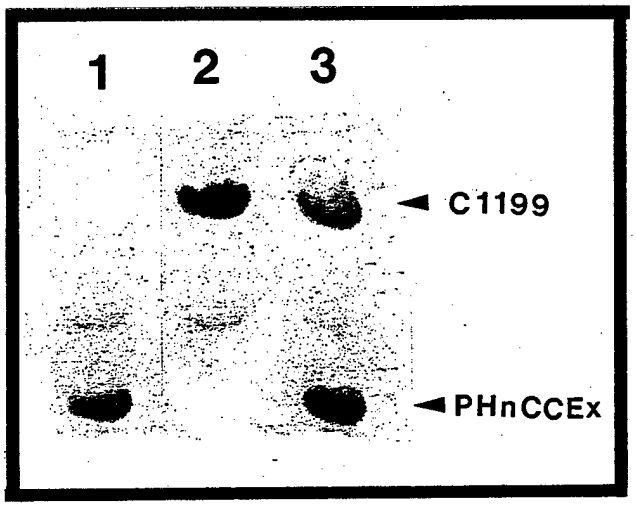

Fig. 8: Transfection of SP1cells with GFP-tagged PHn-CC-ExcDNA or HA-tagged C1199Tiam1cDNA or co-transfection of GFP-tagged Phn-CC-ExcDNA and HA-tagged C1199Tiam1cDNA.
1: Detection of GFP-tagged PHn-CC-Ex expression by anti-GFP-mediated immunoblot.
2: Detection of HA-tagged C1199 Tiam1 expression by anti-HA-mediated immunoblot.
3: Detection of co-expression of GFP-tagged PHn-CC-Ex and HA-tagged C1199 Tiam1 by immunoblotting with anti-GFP antibody followed by anti-HA antibody.

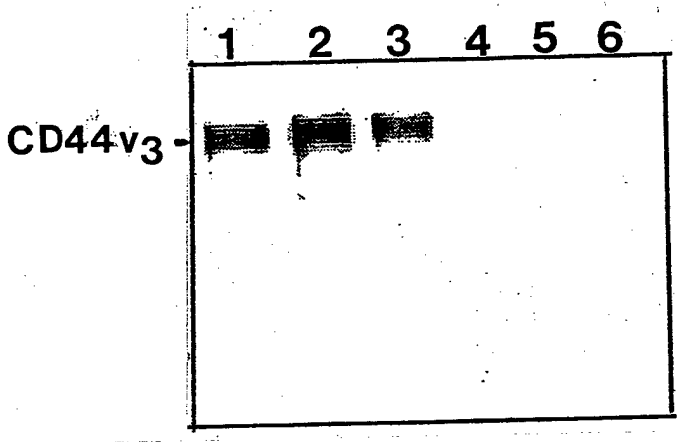

Fig. 9: In vitro binding between CD44v3 and Tiam1-related protein.
CD44v3 was immunoprecipited from surface biotinylated SP1 cells by anti-CD44v3 antibody as described in Material and Methods. Subsequently, purified surface biotinylated CD44v3 was incubated with Tiam1, C1199 Taim1 or PHn-CC-Ex-coated beads in the binding buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% bovine serum albumin and 0.05% Triton X-100) at room temperature for 1 hr. After extensive washing, protein bound on the beads were eluted with glutathione and analyzed with Extravidin (horseradish peroxidase conjugated).
Lane 1: Binding of CD44v3 to Tiam1-conjugated beads.
Lane 2:Binding of CD44v3 to C1199 Tiam1-conjugated beads.
Lane 3:Binding of CD44v3 to PHn-CC-Ex-conjugated beads.
Lane 4: Binding of CD44v3 to Tiam1-conjugated beads in the presence of an excess amount (≈100-fold) of soluble PHn-CC-Ex.
Lane 5:Binding of CD44v3 to C1199 Tiam1-conjugated beads in the presence of an excess amount (≈100-fold) of soluble PHn-CC-Ex.
Lane 6:Binding of CD44v3 to PHn-CC-Ex-conjugated beads in the presence of an excess amount (≈100-fold) of soluble PHn-CC-Ex.